2013

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V. D'Amico
USDA Forest Service, Northern Research Station, vincedamico@gmail.com

J.D. Podgwaite
USDA Forest Service, Northern Research Station

R. Zerillo
USDA Forest Service, Northern Research Station

P. Taylor
USDA-ARS, BIIRL

R. Fuester
USDA-ARS, BIIRL

Journal of Invertebrate Pathology 114 (2013) 158–160

Contents lists available at ScienceDirect

Journal of Invertebrate Pathology

journal homepage: www.elsevier.com/locate/jip

Short Communication

Interactions between an injected polydnavirus and *per os* baculovirus in gypsy moth larvae

V. D'Amico [a,*], J.D. Podgwaite [b], R. Zerillo [b], P. Taylor [c], R. Fuester [c]

[a] USDA Forest Service, Northern Research Station, Department of Entomology & Wildlife Ecology, 531 South College Ave, Newark, DE 19716, United States
[b] USDA Forest Service, Northern Research Station, 51 Mill Pond Rd., Hamden, CT 06514, United States
[c] USDA-ARS, BIIRL, 501 S. Chapel St., Newark, DE 19713, United States

ARTICLE INFO

Article history:
Received 29 January 2013
Accepted 31 July 2013
Available online 8 August 2013

Keywords:
Polydnavirus
Baculovirus
LdMNPV
Lymantria dispar
Insect immunity
CmeBV

ABSTRACT

Larval gypsy moths, *Lymantria dispar* (Lepidoptera:Lymantriidae) were co-infected with the *L. dispar* nucleopolyhedrovirus (LdMNPV) and the *Cotesia melanoscela* (Hymenoptera:Braconidae) polydnavirus (CmeBV). CmeBV was given along with a parasitoid egg and calyx products in a stinging event, or in the form of an injection of calyx-derived extract. LdMNPV was delivered *per os*, integrated into artificial diet. Mortality from all sources was recorded over the subsequent three-week period. Neither parasitism nor injections of purified CmeBV with toxin had any effect on the amount of mortality caused by concurrent challenges with LdMNPV.

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1. Introduction

Polydnaviruses (PDVs) occur in many genera of ichneumonids (*ichnoviruses*) and braconids (*bracoviruses*) (Stoltz and Whitfield, 1992; Webb and Strand, 2005) but replicate only in the ovarian calyx cells of female wasps. The primary function of PDVs is to immunosuppress larvae parasitized by the wasp and overcome encapsulation of parasitoid eggs (Stoltz et al., 1988). Additionally, Washburn et al. (1996) reported that parasitism of *Heliothis zea* larvae via *Campoletis sonorensis* (Hymenoptera:Ichneumonidae) led to immunosuppression that increased spread of the *Autographa californica* nucleopolyhedrosis virus (AcMNPV) in larval tissues. Washburn et al. (1996) theorized that the increased virulence was due to the effects of the *C. sonorensis* polydnavirus on larval hemocytes, which may normally clear early AcMNPV infection in *H. zea* by encapsulating infected cells.

Parasitoids both produce and transmit PDVs, and also have a role in field dissemination of baculoviruses, especially the nucleopolyhedroviruses (NPVs) that infect lepidopteran hosts (Kurstak and Vago, 1967; reviewed by Cossentine, 2009). Our work stems from numerous observations of interactions between a lepidopteran larva (gypsy moth), its baculovirus (LdMNPV), and a braconid parasitoid (*Cotesia melanoscela*). During ovipositionC. *melanoscela* injects the bracovirus CmeBV into larvae along with calyx secretions and an egg (Stoltz et al., 1986). Parasitism by this wasp ranges from 0% to 80% in both samples collected in the field and reared until wasp emergence (Reardon and Podgwaite, 1976; D'Amico et al., 1999), and in larvae exposed to parasitism by *C. melanoscela* concurrently with LdMNPV (Woods and Elkinton, 1987).

Guzo and Stoltz (1985) used CmeBV to immunosuppress *Orgyia leucostigma* to the extent that it became permissive for three parasitoid species that under normal circumstances were unable to complete their life cycle within that host. CmeBV, however, did not make gypsy moth larvae permissive for any of these parasitoids. Lovallo et al. (2002) explored the effects of the *C. congregata* PDV on the immune responses of three species of lepidopteran larvae, including *Lymantria dispar*. One of the striking findings of their work was that despite relatively close phylogenetic relationships between parasitoid–host players, the immune responses of gypsy moth larvae were essentially unaffected by that PDV.

We hypothesized that if CmeBV had the expected immunosuppressive properties of other PDVs, then it would influence gypsy moth disease dynamics by increasing susceptibility to *per os* LdMNPV challenge. Separately both CmeBV and LdMNPV have been studied extensively, but their interactions have not been investigated.

* Corresponding author. Address: U. of Delaware, Dept. of Entomology and Wildlife Ecology, 531 South Ave, Newark, DE 19716, United States. Fax: +1 302 831 8889.
E-mail address: vdamico@fs.fed.us (V. D'Amico).

0022-2011/$ - see front matter Published by Elsevier Inc.
http://dx.doi.org/10.1016/j.jip.2013.07.009

2. Materials and methods

Gypsy moth larvae were from a "New Jersey" strain (US Forest Service, Hamden, CT). *C. melanoscela* adults were reared on gypsy moth larvae at the ARS Beneficial Insects Laboratory in Newark, DE. Larvae were used immediately after molting to the 2nd instar. Using methods similar to Washburn et al. (2000), larvae were challenged *per os* with LdMNPV, and by parasitization or injection with PDV. After challenge they were reared on artificial diet (Bell et al., 1981) for three weeks, and dead individuals examined using light microscopy to verify LdMNPV as the cause of death. Mortality data were compared using a two-factor analysis of variance. Paired *t* tests were used to compare individual points where appropriate, with *P*values of less than 0.05 considered statistically significant.

To explore the effects of *C. melanoscela* parasitism and CmeBV on LdMNPV virulence, a cohort of gypsy moth larvae were given one of two treatments, either a sting from *C. melanoscela* 48 h prior to an LdMNPV dose; or an LdMNPV dose only, delivered at the same time as that given to the stung larvae. Larvae in both treatments were further divided arbitrarily into groups of ~30 larvae and fed cubes of virus-incorporated diet containing 0 (control), or 500, 5,000, 10,000, 20,000 or 50,000 OBs/ml (Slavicek et al., 1992). Only larvae eating the entire cube were used; in any case only 25 larvae were used for each treatment. The effects of CmeBV on larval immunity are known to last at least through the four or five days comprising the early larval development phase of *C. melanoscela* (Stoltz et al., 1986), so we considered our 48 h timing choice to be reasonable. Stung larvae in the 0-dose group were the controls for parasitism, and the unstung 0-dose group for LdMNPV contamination.

We repeated the experiment above with a change in timing of treatments. Two groups of larvae were given either a stung or unstung treatment but larvae fed on contaminated diet starting 48 h *before* being stung, rather than after. We added this treatment pair to see if a change in the order of parasitization by *C. melanoscela* or infection by LdMNPV would have a strong effect on mortality from either agent.

Using methods described by Beckage et al. (1994), an experiment was designed in which CmeBV was extracted from 150 female*C. melanoscela* and then injected into larvae. One wasp equivalent of CmeBV preparation (crude ovarian extract in Grace's medium to ca. 1 μl per injected larva) was injected into each of 150 third instar larvae with a Hamilton syringe and a 31 gauge needle. Another group of 150 larvae were injected with 1 μl of Grace's medium only, and another group of 150 larvae were not injected. Each group was arbitrarily divided further into groups of 25 and dosed with LdMNPV at rates of 0, 500, 5,000, 10,000, 20,000 and 50,000 OBs/ml of diet. Statistical analysis was performed as above.

3. Results

There was no virus-caused mortality in the controls in any experiment. Parasitism was approximately 80% in the controls and was verified on emergence or through dissection of parasitized larvae. Controls are shown for comparison of developmental data. When parasitism occurred 48 h *before* dosing, it did not significantly change the amount of LdMNPV-caused mortality observed in larvae (Fig. 1A) (df = 1, $F = 2.21$, $P = 0.188$), although stung larvae experienced *less* mortality at the two highest doses of LdMNPV.

LdMPNV mortality in larvae stung 48 h after LdMNPV challenge was similar to mortality in larvae stung prior to challenge. Thus, parasitism did not significantly change the amount of LdMNPV-caused mortality observed in larvae (Fig. 1B) (df = 1, $F = 3.194$, $P = 0.124$). In fact, stung larvae experienced *more* mortality at the four highest doses of LdMNPV, although this difference did not produce significant differences in overall mortality at the $P = 0.05$ level.

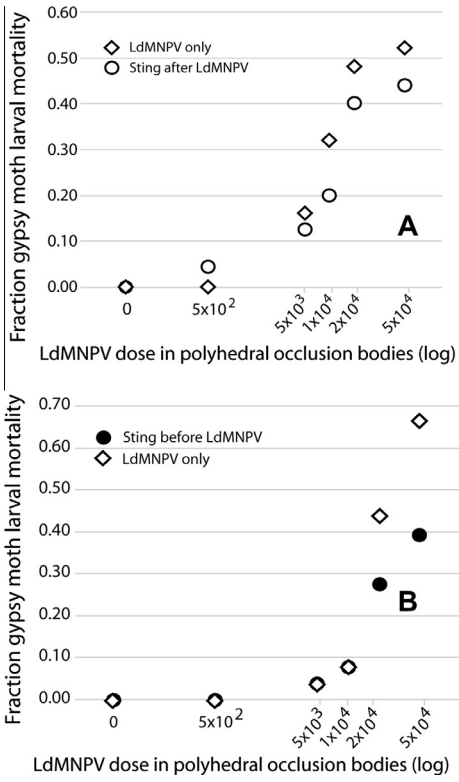

Fig. 1. (A) LdMNPV-caused mortality in gypsy moths stung by *C. melanoscela* as newly emerged 2nd-instar larvae, and dosed with LdMNPV *per os* 48 h later, and unstung larvae given LdMNPV only. LdMNPV doses were administered to all larvae at the same time. There was no LdMNPV mortality in any control larvae (not shown). There were no significant differences between the treatments. (B) LdMNPV-caused mortality in gypsy moths dosed with LdMNPV *per os* as newly emerged 2nd-instar larvae, and stung by *C. melanoscela* 48 h after, and unstung larvae given LdMNPV only. LdMNPV doses were administered to all larvae at the same time. There was no LdMNPV mortality in any control larvae (not shown). There were no significant differences between the treatments.

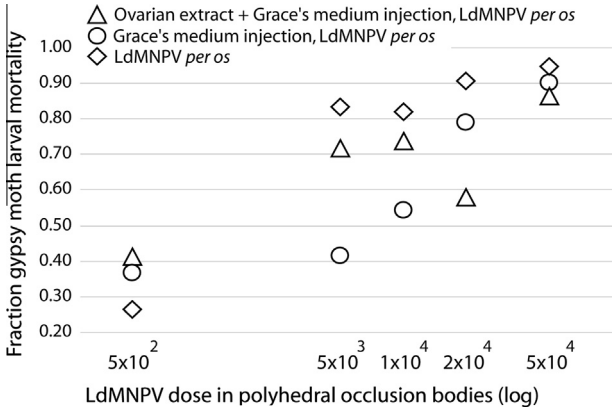

Fig. 2. LdMNPV-caused mortality in gypsy moths treated as newly emerged 3rd-instar larvae with: an extract of *C. melanoscela* ovarian calyx tissue in Grace's medium and LdMNPV *per os*, Grace's medium and LdMNPV *per os*, or LdMNPV *per os*. A two-factor ANOVA performed on the three possible treatment comparisons showed no significant differences between LdMNPV-caused mortality in the larvae treated with CmeBV + Grace's + LdMNPV versus those treated with LdMPV only ($P = 0.114$), or between larvae treated with CmeBV + Grace's + LdMNPV versus those given Grace's + LdMNPV ($P = 0.442$).

Results of our injection experiment (Fig. 2) were consistent with those using parasitoids to inject CmeBV into larvae. The two-factor ANOVA performed on the three possible treatment comparisons revealed no significant differences between LdMNPV-caused mortality in the larvae treated with CmeBV + Grace's + LdMNPV versus those treated with LdMPV only (df = 1, 4.063, $P = 0.114$), or between larvae treated with CmeBV + Grace's + LdMNPV versus those given Grace's + LdMNPV (df = 1, $F = 0.725$, $P = 0.442$). The treatment pair that did not include CmeBV, LdMNPV versus Grace's + LdMNPV, showed significant differences in LdMNPV-caused mortality (df = 1, $F = 8.48$, $P = 0.04$): mortality was higher in larvae not given an injection of Grace's medium at all but the lowest NPV dose (Fig. 2).

4. Discussion

In our experiments we found no effects of parasitism or injection of CmeBV on gypsy moth mortality either before or after *per os* challenge with LdMNPV. This is evidence that immunosuppression produced by CmeBV or other *C. melanoscela*-derived ovarian calyx products does not compromise the defenses with which gypsy moth larvae resist *per os* LdMNPV. While there are no directly comparable studies of CmeBV and LdMNPV, one recent study by McNeil et al. (2010a) showed that intrahemocoelic injections of the *Glyptapanteles flavicoxis* polydnavirus markedly increased levels of infection in gypsy moth larvae after injections of the budded virus form of LdMNPV at 3.750 tissue culture infectious dose ($TCID_{50}$) units, or three to four budded virus particles. Reasons for this result are not yet well-understood.

The earliest stages of the NPV infection process (dissolution of polyhedra, penetration of the peritrophic membrane, or binding of ODV to midgut cells) are likely not subject to influence by concurrent PDV infection. Washburn et al. (1996) strongly implicated the PDV-mediated abrogation of hemocytic encapsulation of infected cells as the reason for a rapid spread of AcMNPV in a "highly refractory" host, *H. zea*. Other work by McNeil et al. (2010b) does not lend strong support for that mechanism achieving the same protective role in the gypsy moth–LdMNPV system. They found strong evidence for some encapsulation and melanization after LdMNPV challenge, but no obvious correlation between these and observed mortality. It may be that the gypsy moth–CmeBV–LdMNPV system is somehow atypical, although we will have to repeat our work, adding budded baculovirus injections and using both *C. melanoscela* and *G. flavicoxis* PDVs, before further conjecture.

Questions raised by this and similar studies emphasize the usefulness of PDVs in unraveling the sequence and mechanisms of insect baculovirus resistance. The continued exploration of interactions between polydnaviruses and baculoviruses, in this system as well as others, is therefore likely to be fruitful on both a microbiological and ecological basis.

Acknowledgments

The authors thank D.B. Stoltz and M.R. Strand for help and advice during the early planning stages of this work, and J. Slavicek for lengthy discussions, comments, and suggestions that were of great help in improving the ms. We thank C. Bartlett for reviewing the ms prior to its submission to *JIP*, and K. Hoover for her review and many helpful comments.

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